ORIGINAL ARTICLE 10.1111/j.1469-0691.2007.01703.x

# Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Majorcan hospitals: high prevalence of the epidemic clone EMRSA-15

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## ABSTRACT

Clinical isolates ($n$ = 389) of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered from 371 patients between January 2003 and June 2004 at the three major public hospitals on the island of Majorca, Spain were studied. The clonal relatedness of MRSA isolates was determined by pulsed-field gel electrophoresis (PFGE) after digestion with *Sma*I. During the study period, MRSA was found in 31% of patients with *S. aureus*-positive cultures. PFGE analysis identified three predominant clones, affecting 94% of the patients. The three clones had been detected since 1999 in one hospital, and were designated as clones A, B and C. Whereas clones A and B (multidrug-resistant) were related to the two most prevalent clones in Spain at this time, clone C was identical to EMRSA-15, currently one of the most common MRSA clones in UK hospitals and also detected in other countries, but rarely in Spanish hospitals. This imported epidemic clone was detected in *c.* 10% of patients admitted to one of the three hospitals in 2002, but its prevalence has increased significantly (32% of the patients investigated in the three hospitals in the present study), and this clone also accounted for 44% of the isolates from non-hospitalised patients. Even though EMRSA-15 showed the least multidrug resistance of the three major clones, it was apparently more virulent, since it was associated significantly (p 0.001) with bacteraemia, and positive blood cultures were documented for 21% of the patients infected by this clone, compared with only 10% and 7% of patients infected with clones A and B, respectively.

**Keywords** Clones, epidemiology, Majorca, methicillin-resistant *Staphylococcus aureus*, pulsed-field gel electrophoresis, virulence

**Original Submission:** 30 August 2006; **Revised Submission:** 16 November 2006; **Accepted:** 19 December 2006

*Clin Microbiol Infect* 2007; **13**: 599–605

## INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were identified soon after the introduction of methicillin into clinical practice [1,2]. Since the first outbreaks of infection caused by MRSA in Europe during the early 1960s [3], this pathogen has spread worldwide, not only throughout the hospital environment, but also among community patients without exposure to healthcare systems. Furthermore, the incidence of MRSA is increasing, despite the development of infection control programmes in many countries. Molecular epidemiology studies have shown that the current MRSA pandemic is the result of the global dissemination of a few highly successful clones [4,5].

In Spain, the first nosocomial outbreak of this pathogen was detected in 1981 [6,7], but MRSA was not a serious problem until the first outbreaks were detected in major Spanish cities at the end of 1989 [8,9]. Most MRSA outbreaks in Spain between 1989 and 1995 were caused by the 'Iberian' clone, which was first detected in 1989 in Barcelona [10], and which showed resistance to most antibiotic groups (i.e., macrolides, tetracyclines, aminoglycosides and quinolones). However, in the mid-1990s, this clone was progressively supplanted by other MRSA clones associated with less multidrug resistance [11,12]. A Spanish surveillance study performed in 2002

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showed a progressive increase in the proportion of MRSA isolates (from 1.5% in 1986 to 31.2% in 2002), and an alarming number of methicillin-resistant isolates were recovered from outpatients; thus, while <5% of community-acquired *S. aureus* isolates in 1994 were methicillin-resistant, 17.8% were methicillin-resistant in 2002 [13].

The first MRSA outbreak on the island of Majorca was described in June 1999; since then the epidemiology of MRSA infections has changed in terms of both clinical and microbiological aspects. The aims of the present work were to analyse recent data concerning the molecular epidemiology and susceptibility of the different MRSA clonal types circulating in Majorcan hospitals, and to compare these results with those of previous local and national studies in Spain, in order to describe the evolution of the epidemiology of this pathogen on Majorca in recent years.

## MATERIALS AND METHODS

### Patients and clinical isolates

The study was carried out between January 2003 and June 2004 in the three main public hospitals that serve nearly all the population of the island of Majorca, Spain: Hospital Universitari Son Dureta (HUSD; 800 beds), which is the tertiary reference hospital of the Balearic Islands; Hospital Son Llàtzer (330 beds); and Hospital de Manacor (200 beds). Only the first clinical isolate of MRSA (isolates from colonisation studies were not included) from each patient was included in the molecular epidemiology study, except for 18 patients who were admitted to two different hospitals at different times, for whom the first isolate from each admission was included. Types of clinical sample and hospital wards were recorded for MRSA-positive patients. Isolates were identified as *S. aureus* by standard microbiological procedures [14].

### Antimicrobial susceptibility testing

Susceptibilities to oxacillin, vancomycin, teicoplanin, ciprofloxacin, erythromycin, clindamycin, gentamicin, mupirocin, fusidic acid, rifampicin and trimethoprim–sulphamethoxazole were determined by disk-diffusion according to CLSI recommendations [15]. The breakpoints used for fusidic acid (susceptible (S) ≥28 mm, intermediately-susceptible (I) 24–27 mm, resistant (R) ≤23 mm) and mupirocin (S ≥14 mm, R ≤13 mm) were those recommended by the disk manufacturer (Rosco Diagnostica, Taastrup, Denmark). The classical phenotypes of macrolide–lincosamide–streptogramin B ($MLS_B$) resistance were defined as follows: constitutive (c)$MLS_B$, erythromycin- and clindamycin-resistant; inducible (i)$MLS_B$, erythromycin-resistant, with clindamycin resistance inducible by erythromycin (presence of antagonism between the two disks); M phenotype, erythromycin-resistant and clindamycin-susceptible (absence of antagonism).

### Molecular epidemiology studies

All MRSA isolates were characterised by macrorestriction analysis of chromosomal DNA after *Sma*I digestion and separation of the fragments by pulsed-field gel electrophoresis (PFGE) using a CHEF-DR III contour-clamped homogeneous electric field apparatus (Bio-Rad Laboratories, Richmond, CA, USA), programmed at 200 V (6 V/cm) for 23 h, with switching times ramped from 1 to 3 s. DNA fragments were visualised by staining with ethidium bromide and photographed under UV illumination, and were then interpreted following criteria recommended previously [16]. Control MRSA strains comprised: the three major clones (designated A, B and C) detected in previous studies at HUSD [17]; the two predominant MRSA clones, designated $P_1$ and $Q_1$, found in a multicentre Spanish study in 2004 [12]; the pandemic Iberian clone (ATCC BAA-44) [18]; the Brazilian clone (ATCC BAA-43) [19]; the Hungarian clone (ATCC BAA-39) [20]; the New York–Japan clone (ATCC BAA-41) [21]; the paediatric clone (ATCC BAA-42) [22]; and two isolates of EMRSA-15 (DEN4561 and HAR22) [23,24]. The reference strain *S. aureus* NCTC 8325 [16] was used as a molecular size standard to normalise the PFGE profiles. The nomenclature for MRSA clones in the present study was based on that established for previous collections [17].

### Data analysis

Statistical analysis of the categorical variables was performed using Fisher's exact test and SPSS software (SPSS Inc., Chicago, IL, USA), with $p < 0.05$ considered to be statistically significant.

## RESULTS

During the period of the study, 389 sequential MRSA isolates (202 from HUSD, 154 from Hospital Son Llàtzer, 33 from Hospital de Manacor) were recovered from 371 patients in the three participating hospitals, representing 31% of the patients with *S. aureus*-positive cultures. For 170 (46%) of the 371 patients, MRSA was isolated from only one sample type, whereas it was isolated from two or more sample types for 201 (54%) patients. The most frequent source of MRSA was exudates, mainly wound infections, ulcers and abscesses (49% of the patients), followed by the respiratory tract (37%). MRSA-positive blood cultures and intravenous catheters were documented for 14% and 7% of the patients, respectively.

Analysis of the PFGE patterns of the 389 MRSA isolates revealed that 366 (94%) belonged to three main clonal types (A, B and C) (Fig. 1). The remaining 23 (6%) MRSA isolates had different PFGE patterns, each being detected in <1.5% of the patients, and were therefore considered to be sporadic clones. Clone A accounted for 132 (34%) isolates, clone B for 109 (28%) isolates, and clone

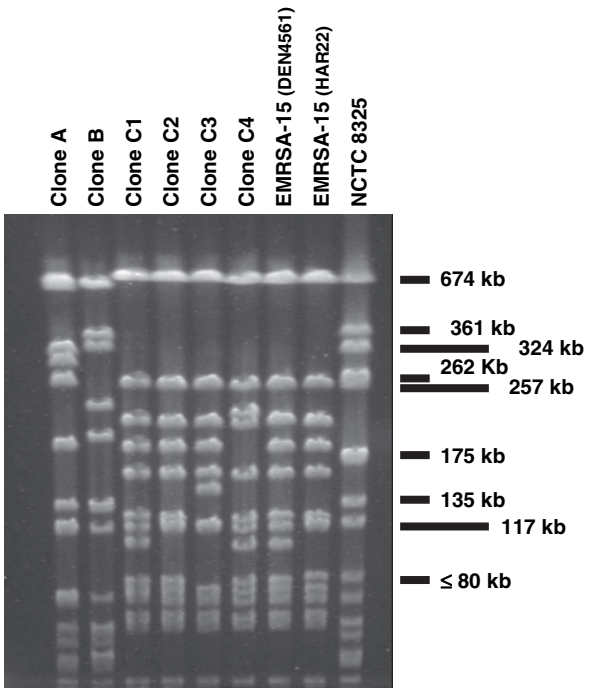


**Fig. 1.** *Sma*I macrorestriction patterns of clone A, clone B and the four subtypes of clone C (C1–C4). Two isolates of EMRSA-15 are included for comparison. Reference strain NCTC 8325 was used as the molecular size standard.

**Table 1.** Distribution of the major methicillin-resistant *Staphylococcus aureus* (MRSA) clones, grouped according to the hospital, hospital department and source of isolation

| | Total MRSA *n* (%) | Clone A *n* (%) | Clone B *n* (%) | Clone C *n* (%) | Others *n* (%) |
|---|---|---|---|---|---|
| Overall | 389 (100) | 132 (34) | 109 (28) | 125 (32) | 23 (6) |
| Hospital | | | | | |
| HUSD | 202 (52) | 63 (31) | 70 (35) | 57 (28) | 12 (6) |
| Son Llàtzer | 154 (40) | 64 (42) | 34 (22) | 51 (33) | 5 (3) |
| Manacor | 33 (8) | 5 (15) | 5 (15) | 17 (52) | 6 (18) |
| Department[a] | | | | | |
| Medical wards | 182 (46) | 69 (38) | 42 (23) | 61 (33) | 10 (6) |
| Surgical wards | 75 (19) | 22 (29) | 30 (40) | 17 (23) | 6 (8) |
| ICU | 43 (11) | 14 (32) | 18 (42) | 11 (26) | 0 (0) |
| Non-hospitalised | 66 (16) | 17 (26) | 14 (21) | 29 (44) | 6 (9) |
| Source of isolation[a] | | | | | |
| Blood | 51 (13) | 13 (25) | 8 (15) | 27 (52) | 3 (5) |
| Respiratory tract | 137 (35) | 53 (38) | 43 (31) | 36 (26) | 4 (2) |
| Exudates | 190 (48) | 61 (32) | 53 (27) | 64 (33) | 12 (6) |

[a]Data for isolates from other or unknown departments ($n$ = 23) and sources of isolation ($n$ = 11) not shown.
HUSD, Hospital Universitari Son Dureta; ICU, intensive care unit.

C for 125 (32%) isolates (Table 1). These major clones were not related to any of the five previously described pandemic clones, including the Iberian clone, but clones A and B have been shown previously to be related to the two most prevalent clones (clones $P_1$ and $Q_1$) found in Spain at the present time. Clone C had a PFGE pattern identical to that of the EMRSA-15 epidemic clone (Fig. 1). Four subtypes of this clone (C1–C4) were detected (Fig. 1); the C1 subtype showed a PFGE pattern identical to that of one of the known EMRSA-15 control isolates (DEN4561), and the C2 subtype was identical to the other control isolate (HAR22).

All three major clones were detected in all three participating hospitals, although with slight differences among the different institutions (Table 1). In HUSD, the most frequent clone was clone B, isolated from 35% of patients with MRSA-positive cultures (p 0.002 compared with the other hospitals, 21%). In contrast, clone A, detected in 42% of patients, was the most frequent clone in Hospital Son Llàtzer (p <0.001 compared with the other hospitals, 29%), and clone C (52%) was the most frequent clone in Hospital Manacor (p <0.001 compared with the other hospitals, 30%).

Several interesting differences were observed when the distributions of MRSA clones from patients on different wards were compared (Table 1). The prevalence of MRSA clone B was significantly higher among patients admitted to intensive care units (ICUs) and surgical wards, being isolated from 42% of all patients with MRSA-positive cultures in ICUs, compared with 26% in non-ICU wards (p 0.003), and from 40% of all patients with MRSA-positive cultures in surgical units (p <0.001). No significant differences were observed in terms of the distribution of clone B in the ICUs of the three participating hospitals, although most (93%) MRSA isolates from ICUs were recovered from just two hospitals. Moreover, the overall high prevalence (47%) of clone B among patients in surgical wards was caused largely by the contribution from a single hospital.

In contrast, clone C (EMRSA-15) was isolated more frequently from non-hospitalised patients (emergency room and outpatient departments), being isolated from 44% of patients with MRSA-positive cultures in these departments, compared with 30% of hospitalised patients (p <0.001), suggesting that this clone is widespread in the community.

Interesting differences were also observed when the different sources of infection were compared (Table 1). Remarkably, the proportion of clone C (EMRSA-15) among blood specimens (52%) was significantly higher (p <0.001) than among other specimens (29%). Moreover, positive

**Table 2.** Antibiotic resistance rates among 389 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from Majorca, Spain

| Antibiotic | Total MRSA (*n* = 389) *n* (%) | Clone A (*n* = 132) *n* (%) | Clone B (*n* = 109) *n* (%) | Clone C (*n* = 125) *n* (%) | Others (*n* = 23) *n* (%) |
|---|---|---|---|---|---|
| Ciprofloxacin | 373 (96) | 128 (97) | 109 (100) | 122 (98) | 14 (61) |
| Gentamicin | 128 (33) | 17 (13) | 101 (93) | 6 (5) | 4 (17) |
| Trimethoprim–sulphamethoxazole | 4 (1%) | 0 | 3 (3%) | 0 | 1 (4%) |
| Vancomycin | 0 | 0 | 0 | 0 | 0 |
| Teicoplanin | 0 | 0 | 0 | 0 | 0 |
| Rifampicin | 9 (2) | 5 (4) | 3 (3) | 1 (1) | 0 |
| Mupirocin | 21 (5) | 10 (8) | 11 (10) | 0 | 0 |
| Fusidic acid | 16 (4) | 6 (4) | 2 (2) | 7 (6) | 1 (4) |
| Erythromycin | 272 (70) | 83 (63) | 104 (95) | 75 (60) | 10 (43) |
| Clindamycin | | | | | |
| $cMLS_B$ | 212 (55) | 78 (59) | 100 (92) | 25 (20) | 9 (39) |
| $iMLS_B$ | 60 (15) | 5 (4) | 4 (4) | 50 (40) | 1 (4) |

$cMLS_B$, constitutive macrolide–licosamide–streptogramin B ($MLS_B$) resistance; $iMLS_B$, inducible $MLS_B$ resistance.

blood cultures were documented for 21% of the patients who were positive for this clone, compared with 9% of patients positive for other clones (p 0.001). The percentages of patients with positive blood cultures who were infected by the two other major clones (A or B) were only 10% and 7%, respectively.

Table 2 shows the antimicrobial resistance rates among all 389 MRSA isolates, as well as for the different clonal types. Overall, the MRSA isolates were uniformly susceptible to vancomycin and teicoplanin, and showed low rates of resistance to mupirocin (5%), fusidic acid (4%), rifampicin (2%) and trimethoprim–sulphamethoxole (1%). However, since disk-diffusion was used to test susceptibility, the presence of isolates with low-level glycopeptide resistance cannot be ruled out. Most (96%) isolates were resistant to ciprofloxacin, whereas resistance to erythromycin (70%), clindamycin (55% with $cMLS_B$; 15% with $iMLS_B$) and gentamicin (33%) was more variable. Clone B was associated with the highest rates of multiresistance, showing 100% resistance to ciprofloxacin (p 0.004 compared with other clones), 95% to erythromycin (p <0.001), 92% to clindamycin, plus an additional 3% with the $iMLS_B$ phenotype (p <0.001), and 93% to gentamicin (p <0.001). In contrast, clone C (EMRSA-15) showed the lowest rates of multiresistance among the three major clones, and a significantly lower level (5%) of resistance to gentamicin (p <0.001). Interestingly, clone C frequently showed the $iMLS_B$ phenotype (40% of all clone C isolates, including 67% of those resistant to erythromycin), whereas this phenotype was infrequent among the other clonal types (4% of all the non-clone C isolates, including 5% of those resistant to erythromycin) (p <0.001).

## DISCUSSION

During the last four decades, methicillin resistance in *S. aureus* has been a problem of global dimensions, affecting mainly hospitalised patients, although MRSA has also emerged as a community-acquired pathogen in recent years. The prevalence of MRSA in Europe follows a north-to-south gradient, being lowest in Scandinavian countries (<2%) and highest in southern Europe, e.g., Greece, Italy, France, Spain and Portugal (30–60%) [25]. In Spain, the prevalence of MRSA has increased continuously since 1986 (1.5%), reaching 31% in 2002 [13]. Also, as in other countries, MRSA infections originating in the community are no longer infrequent, accounting for 18% of community-acquired *S. aureus* infections during 2002 [13].

Although the prevalence (31%) of MRSA revealed in Majorcan hospitals by this study is very similar to that reported in the rest of Spain, the molecular studies revealed remarkable differences concerning the epidemiology of this pathogen on the island. In addition to the two predominant MRSA clones found in Spanish hospitals, a high prevalence (32%) of the epidemic clone EMRSA-15 was found. Clone C, now classified as EMRSA-15, was already present in Majorca in 1999 (10% of MRSA-positive patients), but its prevalence has increased dramatically in recent years, so that it accounted for almost one-third of patients with MRSA who attended Majorcan hospitals in the present study. EMRSA-15 is the most frequent clone isolated in UK hospitals [26], and has also been detected in Germany [27], Finland, Sweden, Belgium [28], Portugal [29], Australia [30] and New Zealand [31]. This clone was reported for the first time in Spain at a teaching hospital in Tenerife (Canary Islands) [32], but the overall prevalence of EMRSA-15 in Spanish hospitals was found to be low (0.7%) in a large study involving >2000 MRSA isolates recovered between 1996 and 2002 from 110 Spanish hospitals [11].

The differential high prevalence of EMRSA-15 in Majorcan hospitals may be linked to the fact that the island is a frequent tourism destination (as is the Canary Islands) for individuals from countries with a high prevalence of this clone,

which perhaps highlights the importance of travel in the international spread of multiresistant pathogens. The prevalence of EMRSA-15 was even higher among non-hospitalised patients (44% vs. 30%), suggesting that, particularly for this clone, the flux was mainly from the community to the hospital setting, and not the other way around (as might be expected). The increase in MRSA infections in healthcare institutions other than hospitals could also contribute to this inverted flux. A study of patients with decubitus ulcers in a geriatric institution in Majorca between January 2000 and June 2002 revealed a very high prevalence of MRSA (70%), and the proportion of clone C (EMRSA-15) was already high (30%) during that period compared with 10% in the hospital setting [33].

A further interesting finding associated with EMRSA-15 is that, despite showing the lowest frequency of multidrug resistance among the three major clones, it was apparently more virulent, since it was associated significantly with bacteraemia. Positive blood cultures were documented for 21% of the patients infected by this clone, compared with <10% for the other major clones. Analysis of the interplay among pathogenicity, epidemicity and antibiotic resistance is a key element in understanding the evolution of microbial pathogens [34]. Several lines of evidence support the hypothesis of an inverse relationship between antibiotic resistance and epidemicity and pathogenicity in MRSA. Displacement of multidrug-resistant MRSA clones by more susceptible lineages has been described in recent years and, particularly for gentamicin resistance, has been found to be linked with increased fitness of the susceptible lineages and a reduction in the use of gentamicin [12,35–38]. The type of staphylococcal chromosomal cassette (SCC) *mec* complex could also play a role, since the SCC*mec* type showing the most efficient dissemination (SCC*mec*IV) is also associated with reduced antimicrobial resistance [39,40]. Both gentamicin susceptibility (as shown in this study) and the presence of SCC*mec*IV [5] are characteristics of EMRSA-15. Nevertheless, the possible association between EMRSA-15 and bacteraemia, and whether this is related to reduced antimicrobial resistance, needs to be confirmed in clinical studies designed with this specific aim. There are important clinical implications, in that bacteraemia caused by MRSA is associated with increased mortality compared with that caused by methicillin-susceptible strains [41].

In conclusion, widespread dissemination of the epidemic EMRSA-15 clone was found throughout Majorca, and this clearly differs from the situation documented in mainland Spanish hospitals. In addition, a statistically significant association was found between this clone and lower antimicrobial resistance rates, non-hospitalised patients and bacteraemia.

## ACKNOWLEDGEMENTS

We are grateful to H. de Lencastre and A. Tomasz for providing the five pandemic MRSA clones and the two isolates of the EMRSA-15 clone, and to M. Á. Domínguez and C. Borraz for the control strains of clones P and Q. We would also like to thank A. Vindel for sharing unpublished information concerning the epidemiology of MRSA in Spain. This work was supported, in part, by the Red Española de Investigación en Patología Infecciosa (REIPI) from the Fondo de Investigaciones Sanitarias (FIS C03/014), Ministerio de Sanidad y Consumo, Spain.

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